EFFECTS OF CHRONIC LOW DOSE ORGANIC ARSENIC EXPOSURE ON THE LIVER OF SPRAGUE DAWLEY RATS

BY

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ABSTRACT

Monosodium methylarsonate (MSMA) is a potent organoarsenical herbicide that is still being used in most Asian countries, despite its restriction in some other countries. Organic arsenic has been given less attention as it thought to be less toxic than inorganic counterpart. In most studies, the reported adverse effects were mainly on gastrointestinal system with little information on its severity to the liver. The objective of this study was to investigate the effect of organic arsenic (MSMA) exposure on the liver. Sixty rats were divided into three groups with different duration of exposure. The rats were given MSMA at 63.20 mg/kg daily for 2, 4 and 6 months through oral gavage. Serum samples were analysed for AST, ALT and ALP. Arsenic accumulation measurement, histomorphometric evaluation (H&E, PAS, reticulin and TUNEL staining) and ultrastructural study (scanning and transmission electron microscopy) were done on liver tissue. LSEC were isolated for gene expression study. Accumulation of arsenic were significantly higher in the MSMA-exposed rats compared to their control with the highest in the 6-month group [2-month (3.97± 2.28, p=0.009), 4-month (4.57±0.47), p<0.001 and 6-month (21.33±9.83, p=0.004) µg/g]. Both ALT [Control: 85.3± 13.0, Exposed: 52.0±5.2, p=0.005] and ALP [Control: 237.6±52.8, Exposed: 162.9± 28.9, p=0.007] were significantly lower in 4-month MSMA-exposed group than their control.The difference in AST level in all groups were not significant. Histopathological and ultra-structurally, focal necrotic, apoptotic and fibrotic changes in the liver with the reduction of organelles in hepatocytes were observed in 4- and 6-month exposed rats. In 4-month exposed group, the liver displayed increased in ballooning degeneration of the hepatocytes at zone 2, focal necrosis with minimal inflammatory infiltrates with fibrosis (mixture of stage 1 and 2). Disrupted hepatic cords with hepatocytes blebs were seen. In 6-month exposed rats, more extensive changes were noted. Cell cycle, apoptotic and DNA repair gene were affected in this exposure. At 2-month, cell cycle (*Tp53*), apoptotic (*Tnfrsf1a*) and DNA repair (*Xrcc1*) genes showed downward trend. However, at 4-month, both apoptotic-gene (*Bax, Tnfsrf1a and Caspase 2*) and the DNA repair gene (*Xrcc1*) expression showed upward trend. At chronic (6-month) exposure, only DNA repair gene (*Mpg*) showed upward trend. In conclusion, chronic MSMA exposure could be associated with potential liver injury. Thus, long term exposure to MSMA-contaminated water source should be taken seriously.

خلاصة البحث

ميثيلارسونات أحادي الصوديم (MSMA) هو مبيد أعشاب عضوي قوي السينيك لا يزال يستخدم في معظم البلدان الآسيوية ، على الرغم من القيود المفروضة عليه في بعض البلدان الأخرى. تم إعطاء الزرنيخ العضويي اهتماما أقل في معظم الدراسات ، لأنه يعتقد أنه أقل سمية من نظيره غير العضوي. معظم الدرسات اثبتت تأثيره الضارخصيصا علي الجهاز الهضمي, مع القليل من المعلومات حول شدتها على الكبد. الهدف من الدراسة هو دراسة تأثيرالزرنيخ العضوي علي الكبد. ستون فأر قسمت الي ثلاثة مجموعات مع فترات تعرض مختلفة. اعطيت الفئران 63.20 (MSMA) ملي جرام يوميا لمدة اتنين, وأربعة, و ستة اشهرعن طريق تزقيمة الفم. تم تحليل عينات المصل لأجل (ALT, AST). تم أجراء قياس تراكم الزرنيخ, تقييم هستومورفومترك باستخدام صبغات مثل الهيما توكسلين,مانسون تلاتية الالوان, و رتكولين. ودراسة البنية التحتية (المسح المجهري الألكتروني المنتقل) علي انسجة الكبد. تم عزل الخلايا (LSEC) لدراسة التغيرات الجينية. تراكم الزرنيخ كان مرتفع بشكل ملحوظ في المجموعات التي تعرضت له مع اعلي مستوي كان في مجموعة ستة أشهر.في شهرين كانت(3.97±2.28, P=0.009). أربعة أشهر(0.470±4.57, P>0.001) . وستة أشهر(21.33±.9, P=0.004) ميكروجرام\ جرام. كان كل من (ALT) (قبل 66.86±7.6, وبعد 52.5±5.16, P=0.003) و AST (قبل 240.71±52.85, وبعد 162.8±28.86, P=0.001). كانت المستويات اقل بكثير في مجموعة الاربعة أشهر مابين قبل وبعد التعرض. لم يكن الاختلاف في (AST) كبيرا بن المجموعات. لوحظت تغيرات نسيجية مرضية وهيكلية فائقة البؤرة والنخرية والتليقية في الكبد مع نقص العضيات في خلايا الكبد في فئران لمدة 4 و6 أشهر.أظهر الكبد زيادة في التنكيس المنتفخ لخلايا الكبد في المنطقة 2, ونخر بؤري مع الحد الادني من تسلل الألتهاب مع التليف (خليط من مرحلة 1 و 2). شوهدت الحبال الكبدية المعطلةبفقعات خلايا الكبد. داخل الخلايا, لوحظ تفكك السيتوبلازم مع فقدان الشكل العضيات الطبيعي. في الفئران المعرضة لمدة 6 أشهر, لوحظت تغيرات أكثر شمولا. تاثرت دورة الخلية. وجين إصلاح موت الخلايا المبرمج والحمض النووي في هدا التعرض. في شهرين, تم تقيل تنضيم دورة الخلية(Tp 53), والاستماتة (Tnfrsf 1a) وإصلاح الحمض النووي(Xrcc1) بشكل كبير(P>0.005). ومع ذلك, في غضون 4 أشهرتم تنظيم كل من الجين المبرمج (Bax, Tnfsrf1a and Caspase 2) وجين إصلاح الحمض النووي (P>0.005) (CCRX 1). عند التعرض المزمن (لمدة 6 أشهر), تم تنظيم جين إصلاح الحمض النووي فقط (Mpg) بدرجة عالية (P>0.005). في الختام يمكن أن يرتبط التعرض المزمن ل MSMA. بإصابة الكبد المحتملة بالتغيرات الجينية في LSEC. وبالتالي, يجب أن وُخد التعرض الطويل الأمد لمصدر المياه الملوث ب MSMAعلي محمل الجد.

APPROVAL PAGE

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DECLARATION

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This thesis is dedicated to both abah; Saharudin bin Nik (2nd Aug 1949 - 8th November 2018) and Md Supian Abdullah (21st Sept 1945 - 3rd August 2015) who have not managed to see me reaching the finishing line today. I'll tell you when I meet you there, please wait for me, Al-Fatihah. Thank you.

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LIST OF ABBREVIATIONS

$^{\Delta\Delta}C_T$	Delta delta threshold cycle
ADP	Adenosine diphosphate
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANA	Anti-nuclear antibody
AS3MT	Arsenic methyltransferase
As^{III}	Arsenite
AST	Aspartate transaminase
ATP	Adenosine triphosphate
BER	Base excision repair
CAV	Caveolin
cDNA	Complementary DNA
CT/C_T	Threshold cycle
dH_2O	Distilled water
DMA	Dimethylarsinic acid
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorter
GSH	Reduced glutathione
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HepG2	Human liver cancer cell line
HMDS	Hexamethyldisilazane
HNO_3	Nitric acid
HSC	Hepatic stellate cells
ICPMS	Inductive coupled plasma mass spectrometry
IMR-90	Human fetal lung cells
IVC	Inferior vena cava
JNK	c-jun-N-Terminal Kinase Inhibitors
KTX	Ketamin-Xylazine-Zoletil mixture
LDH	Lactate dehydrogenase

LDL	Low-density lipoprotein
LSEC	Liver sinusoidal endothelial cells
MAPK	Mitogen-activated protein kinase
MMA	Monomethylarsinic acid
MMR	Mismatch repair
MnSOD	Mitochondrial antioxidant manganese superoxide dismutase
MSMA	Monosodium methylarsonate
mtTFA	Mitochondrial transcription factor A
NADH	Nicotinamide adenine dinucleotide
NAFLD	Non-alcoholic fatty liver disease
NER	Nucleotide excision repair
NPC	Non-parenchymal cell
NRF-1	Nuclear respiratory factor 1
PARP1	Poly-ADP-ribose polymerase-1
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PECAM	Platelet endothelial cell adhesion molecule
PON1	Paraoxonase-1
PT	Portal triad
Rac1	Ras-related C3 botulinum toxin substrate 1
RIN	RNA integrity number
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute (culture media)
SAM	S-adenosylmethionine
SD	Standard deviation
SEM	Scanning electron microscope
SPC	Sinusoidal progenitor cell
TEM	Transmission electron microscope
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
XPA	Xeroderma pigmentosum group A
XPC	Xeroderma pigmentosum group C-complementing protein

CHAPTER ONE

INTRODUCTION

2.1 BACKGROUND OF THE STUDY

Arsenic is a metalloid found ubiquitously on earth and exists in two forms which are inorganic and organic (Abdul et al., 2015). Over the decades, attention has been given more to the hazardous effects of inorganic arsenic on human health rather than the organic counterpart (Mie et al., 2017; Pateriya et al., 2020). Numerous studies have proven the high association of its exposure to skin cancer, cancer of the internal organs (urinary bladder, kidney, lung and liver), diabetes, high blood pressure and respiratory, circulatory and reproductive disorders (Agarwal et al., 2009; Bhattacharjee et al., 2013). It is estimated that 200 million people worldwide have been exposed to arsenic drinking water above the recommended limit of 10 µg/L, primarily as a result of their contaminated groundwater sources which are located in a naturally high occurring arsenic (WHO, 2017). Majority of the population exposed to arsenic lives in southern Asian countries such as Bangladesh, Cambodia, India, Nepal And Vietnam. Elevated levels of arsenic have also been found in several western countries such as Germany, United Kingdom, USA and Canada (Chung et al., 2014; George et al., 2014; Nordstrom, 2002). In these countries, the rising demands for sanitary water often cannot be met by surface water supplies prompting the focus to the use of ground-water sources.

Organic arsenic exposure, on the other hand, may come from diet, contaminated livestock (Sarkar et al., 2014) and agricultural and industrial area (George et al., 2014). Diet is one of the increasing concern of a non-drinking-water source of arsenic. It is present in a wide varieties of fish and rice. Fish were found to have high amount of

organic arsenic compounds predominantly arsenobetaine (Molin et al., 2015) while rice contains predominantly inorganic arsenic (Jackson et al., 2012). Dust, soil and air have also been a potential contamination risk from arsenic exposure particularly near former mining sites, smelting and industrial areas (Beamer et al., 2014; Menka et al., 2014). Migration of arsenic from sediments and soils to groundwater sources and crops has been believed to be the mechanism of contamination (Carlin et al., 2016) but it is still not well understood and requires more future research.

Organic arsenic has been thought to be less toxic as it is normally believed from previous evidence that it does not remain in the body and expelled more rapidly than the inorganic (Shi et al., 2004; Valko et al., 2006). Upon ingestion, both types leave the body through urine after several days and some even longer. Nonetheless, proof of organic arsenicals hazardous effects is slowly emerging even though the studies were only limited to a certain body system. For instance, keratosis was observed in female workers in a chemical plant who were exposed to 0.065 mg/m^3 arsanilic acid (Yang et al., 2007) and development of erythematous lesions on the feet and ears of rats were found when rats exposed to 6 mg/m^3 dimethylarsinic acid (DMA). Ingestion of 80 mg/kg of organic arsenicals also has been shown to cause vomiting, abdominal pain, hyperactive bowel and diarrhoea (Lee et al., 1995). In a more severe case, accidental ingestion of pasture sprayed with monosodium methylarsonate (MSMA) caused intense diarrhoea and dehydration after grazing in 200 cattle which subsequently led to the death to 16 of the animals (Gonçalves et al., 2017). Previous animal studies have reported that exposure of repeated monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) caused diffuse inflammation and hepatocellular degeneration (Jaghabir et al., 1989), decrease in absolute liver weight (Siewicki, 1981) and reduced liver glutathione, cytochrome P-450 content and serum ornithine

decarboxylase activity (Ahmad et al., 1999). Other than that, several oral doses of roxarsone in pigs have caused significant neurotoxicity with time-dependent degeneration of myelin and axons (Kennedy et al., 1986). To the best of our knowledge, studies on the effect of organic arsenicals on the liver are still rarely documented in the animal model and as well as human studies, prompting the need to explore further in this area.

Toxicity of arsenic depends on the valence state and the species. In rank orders, trivalent arsenite is more toxic than pentavalent arsenate (Hong et al., 2014) and inorganic arsenic species are more toxic than its organic forms (Sarkar & Paul, 2016). Metabolism of the arsenic in the body plays a decisive role in determining the toxicity further (Sarkar & Paul, 2016). Arsenic gets into the body in various ways. Oral ingestion is the commonest route of entry followed by inhalation and dermal absorption. Once it enters, arsenic will generally get methylated; mostly in the forms of MMA and DMA in body cells. These methylated products have cytotoxic and genotoxic effects (Mie et al., 2017; Pateriya et al., 2020) and thus duration and concentration of exposure could play as additional factors in aggravating the deleterious effects.

Several studies have documented the toxicity effects from a different duration of exposure. Acute to subacute (up to 28 days) exposure to inorganic and organic arsenic does not cause significant accumulation in rats' kidney and liver (Lewchalermvong et al., 2018). Findings on the toxicity effect of organic exposure on different duration have been mixed. In a study by Yi et al. (2018a), sub-chronic exposure of arsenic-containing traditional Chinese herbal medicine (realgar) demonstrated significant accumulation of DMA in the liver without changes in liver enzymes and any significant histopathological changes in the organs. Nonetheless, in another study of acute exposure of realgar, glomerulus injury and mild liver injury in rats were observed even the

exposure of the toxicant for only two weeks (Luo et al., 2017). In beagle dogs, exposure to realgar for four weeks produced obvious vomiting, diarrhoea and even death (Zhang et al., 2011). In these studies, however, the dosage used was higher than the actual human exposure and were tested with a different arsenic concentration on a different animal model. There is still lacking evidence on the effect of chronic organic arsenic exposure that reflects the actual duration of human exposures and human-relevant dose.

The liver is an extremely important organ housing many pivotal metabolisms to ensure bodily homeostasis. It is also a well-known target organ of arsenic toxicity. Hepatocytes are metabolically active parenchymal cells that dominate 80% of the liver. Sporadic vacuolation of hepatocytes, sinusoidal dilation (Bhattacharya et al., 2012), hepatocellular degenerative lesions along with inflammatory cells and irregular hepatic cells (Chandranayagam et al., 2013) were among reported findings indicating the capability to induce hepatotoxicity. However, it is not known whether MSMA affects the liver as in inorganic arsenic.

Liver sinusoidal endothelial cells (LSEC), on the other hand, comprised 50% of the non-parenchymal group (Werner et al., 2015). These cells line the liver sinusoids pose an open pore system which facilitates the transfer of substrates between blood and the liver parenchyma. The role of LSEC is currently not fully understood and received growing attention (Deleve, 2013). Perturbation of the LSEC pores affects greatly the substance transfer between blood and surrounding cells and subsequently signals a multitude of liver injury mechanisms such as losing their protective properties (Poisson et al., 2017; Tanoi et al., 2016), angiogenesis (Bocca et al., 2015; Elpek, 2015) and fibrotic process (Deleve, 2015; Poisson et al., 2017). Angiogenesis is an important preceding event associated with the fibrogenic progression of chronic liver diseases.

Since responses vary widely depending on the cell type, arsenic species, length and dose of exposure, it is not known how MSMA would affect LSEC (Deleve, 2015).

This study aimed to investigate the effect of organic arsenic, monosodium methylarsonate (MSMA) exposure on the hepatocytes and LSEC DNA repair system. The research provides additional evidence on the notorious effects of organic arsenic as well as opening more platform to understand the possible mechanism of organic arsenic toxicity through disturbance of LSEC.

2.2 STATEMENT OF THE PROBLEM

i. Organic arsenic was previously thought to be less toxic than inorganic arsenic with most studies focussing on acute and sub chronic exposure.
ii. However, human is more likely to be chronically exposed to organic arsenic through consumption of contaminated ground water sources.
iii. Recent evidence showed that the exposure to chronic organic arsenic could also be as toxic as inorganic arsenic particularly to the gastrointestinal system but little is known about it effects on the liver.
iv. Hepatocytes are liver parenchymal known to be susceptible to the toxic effect of arsenic while LSEC poses as potential site of liver injury.
v. Liver is an important organ for metabolism of various metabolites and a well target organ for arsenic toxicity, this study aim to study the effect of organic arsenic MSMA exposure on hepatocytes and to explore further on other liver potential site of injury.

2.3 RESEARCH OBJECTIVES

2.3.1 General Objective

To investigate the effect of chronic low dose organic arsenic, monosodium methylarsonate (MSMA) exposure on the liver.

2.3.2 Specific Objective

i. To measure total arsenic concentration in the liver of MSMA-exposed rats.
ii. To compare the level of liver enzymes (ALT, AST and ALP) between MSMA-exposed and non-exposed rats.
iii. To determine histopathological changes of liver in MSMA-exposed rats.
iv. To determine ultrastructural changes of liver in MSMA-exposed rats.
v. To assess the gene expression of related apoptosis-regulating gene and DNA repair genes in the MSMA-exposed rats.

2.4 HYPOTHESIS

i. Arsenic is highly accumulated in the liver of MSMA-exposed rats.
ii. Liver enzymes (AST and ALT) are significantly higher in rats exposed to MSMA.
iii. MSMA exposure induces histopathological changes in the liver of MSMA-exposed rats.
iv. MSMA exposure induces ultrastructural changes to organelles in hepatocytes and LSEC of MSMA-exposed rats.
v. The apoptotic-regulating and DNA repair gene expression are altered in MSMA-exposed rats.